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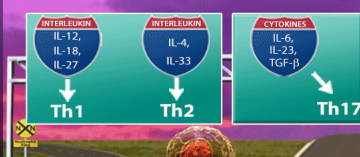
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## Phosphorothioate Backbone Modification Modulates Macrophage Activation by CpG DNA

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# Phosphorothioate Backbone Modification Modulates Macrophage Activation by CpG DNA<sup>1</sup>

David P. Sester, Shalin Naik, Shannon J. Beasley, David A. Hume, and Katryn J. Stacey<sup>2</sup>

Macrophages respond to unmethylated CpG motifs present in nonmammalian DNA. Stabilized phosphorothioate-modified oligodeoxynucleotides (PS-ODN) containing CpG motifs form the basis of immunotherapeutic agents. In this study, we show that PS-ODN do not perfectly mimic native DNA in activation of macrophages. CpG-containing PS-ODN were active at 10- to 100-fold lower concentrations than corresponding phosphodiester ODN in maintenance of cell viability in the absence of CSF-1, in induction of NO production, and in activation of the IL-12 promoter. These enhancing effects are attributable to both increased stability and rate of uptake of the PS-ODN. By contrast, PS-ODN were almost inactive in down-modulation of the CSF-1R from primary macrophages and activation of the HIV-1 LTR. Delayed or poor activation of signaling components may contribute to this, as PS-ODN were slower and less effective at inducing phosphorylation of the extracellular signal-related kinases 1 and 2. In addition, at high concentrations, non-CpG PS-ODN specifically inhibited responses to CpG DNA, whereas nonstimulatory phosphodiester ODN had no such effect. Although nonstimulatory PS-ODN caused some inhibition of ODN uptake, this did not adequately explain the levels of inhibition of activity. The results demonstrate that the phosphorothioate backbone has both enhancing and inhibitory effects on macrophage responses to CpG DNA. *The Journal of Immunology*, 2000, 165: 4165–4173.

Since the original descriptions of the immunostimulatory actions of unmethylated CpG motifs in nonmammalian DNA, there has been a widespread recognition of the implications for gene and antisense therapy, DNA vaccination, and immunotherapy (reviewed in Refs. 1–3). Whereas in DNA vaccines the active moieties that contribute adjuvant activity are native dsDNA molecules, most other applications of CpG DNA have used nuclease-resistant phosphorothioate-modified oligodeoxynucleotides (PS-ODN).<sup>3</sup> PS-ODN have sulfur substituted for one of the nonbridging oxygens in the phosphodiester backbone. Phosphorothioate-stabilized CpG DNA is an effective adjuvant in vaccinations (4–7) as well as having potential in immunotherapy of inflammatory and allergic disease (8–10).

Phosphodiester oligodeoxynucleotides (PO-ODN) containing CpG motifs mimic the direct effects of native bacterial DNA in vitro, including activation of macrophages, dendritic cells, and B lymphocytes. The short  $t_{1/2}$  of PO-ODN in vivo (11) precludes their use in therapy. It is relevant to ask whether the more stable PS-ODN mimic all of the actions of native phosphodiester CpG DNA. Recognition of the differences, and their molecular basis, may direct the identification of more effective mimetics of bacterial DNA for clinical application. There have been few comparative studies of CpG PS- and PO-ODN. Published observations vary

depending on the cell system used and the effector function measured. A CpG PS-ODN was effective at promoting survival of human dendritic cell precursors from peripheral blood at lower concentrations than a CpG PO-ODN (12), but the length and sequence of the respective PS-ODN and PO-ODN were not identical. Studies by Boggs et al. (13) using sequence identical PS-ODN and PO-ODN demonstrated that for NK cell activation PO-ODN were less active than corresponding PS-ODN, but the situation was reversed when a transfection reagent, lipofectin, was added. Several studies indicate that PS-ODN are more potent than corresponding PO-ODN at inducing B cell proliferation (14–17). Studies on B cell proliferation are complicated by the fact that human and mouse B cells can respond to PS-ODN in a CpG-independent manner (18, 19). In addition, if using thymidine uptake as a measure of proliferation, PO-ODN degradation can dilute the radioactive thymidine pool (20). PS-ODN have also been found to be more potent in induction of spleen cell IL-12 and CD69 expression (15).

CpG DNA mimics many of the actions of bacterial LPS on murine macrophages, including activation of NF- $\kappa$ B-dependent transcription and release of cytokines such as TNF- $\alpha$ , IL-6, and IL-12 (21–25). In this study, we use a range of assays of macrophage activation to compare the actions of PS-ODN and PO-ODN of identical sequence. The results show that the two types of ODN have distinct activities and PS-ODN do not function merely as a more stable version of active PO-ODN.

## Materials and Methods

### Cells and reagents

The murine macrophage-like cell line, RAW264, was obtained from the American Type Culture Collection (Manassas, VA). Bone marrow-derived macrophages (BMM) were obtained by cultivation of femoral bone marrow cells for 1 wk on bacteriological petri dishes in recombinant human macrophage CSF (CSF-1) (a gift from Chiron, Emeryville, CA), as described previously (26). The medium in all experiments was RPMI 1640 plus 10% heat-inactivated FBS with 20 U/ml penicillin and 20  $\mu$ g/ml streptomycin. Serum was screened for low LPS contamination using RAW264a4 cells. RAW264a4 is a stable transfectant of RAW264 with an integrated HIV-1 LTR-luciferase reporter gene that is activated by low concentrations of LPS, or CpG DNA (21, 27).

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<sup>3</sup> Abbreviations used in this paper: PS-ODN, phosphorothioate-modified ODN; BMM, bone marrow-derived macrophage; ERK, extracellular signal-regulated kinase; LTR, long terminal repeat; MAP, mitogen-activated protein; ODN, oligodeoxynucleotide; PO-ODN, phosphodiester ODN.

PO-ODN and PS-ODN were purchased from Geneworks (Adelaide, Australia) or Oligos Etc. (Wilsonville, OR). AO-1, a PO-ODN, has the sequence 5'-GCTCATGACGTTCTGATGCTG-3', which is similar to the oligo 1668 of Yi et al. (28), with the addition of terminal G residues. NAO-1 is the same sequence with the core CG (underlined) reversed to GC. AOS-1 and NAOS-1 are the corresponding PS-ODN. AAC-22 (29) is slightly less active than AO-1 and has the sequence ACCGATAACGTTGCCGGTGACG. SAAC-22 is the phosphorothioate version of AAC-22. Results obtained with AO-1 and AAC-22 or their corresponding phosphorothioate versions were similar and they could be used interchangeably in this work. For uptake experiments, AO-1 and AOS-1 were purchased with a Cy3 fluorophore attached at the 5' end. LPS from *Salmonella minnesota* Re595 was purchased from Sigma (St. Louis, MO) and was dissolved as a 10 mg/ml stock solution in 0.1% triethylamine and sonicated. Fluorescein-labeled anionic dextran, 10,000 m.w., was purchased from Molecular Probes (Eugene, OR).

#### Luciferase assays

For the HIV-1 LTR assay,  $2.5 \times 10^5$  RAW264a4 cells were placed in 1 ml of medium in a 24-well plate overnight. After reduction of the total volume to 0.5 ml, cells were incubated with stimuli for 2 h, unless stated otherwise. Cells were harvested and assayed for luciferase activity, as described (21, 27), except that the assays were conducted using a Trilux plate luminometer and reagents provided by Packard Instruments (Meriden, CT). For analysis of IL-12 promoter activity, the murine IL-12 p40 promoter, as described by Cowdery et al. (25), was amplified by PCR and subcloned into the pGL2 luciferase vector by Dr. E. Gold (University of Washington School of Medicine, Seattle, WA). RAW264 cells were transfected with 10  $\mu$ g of pGL2-IL12p and 2  $\mu$ g of the selectable plasmid pNeo/Tak (30), as described (31), and subjected to selection in 200  $\mu$ g/ml G418 for 2 wk. Approximately 200 colonies appeared on the dishes. These were harvested and pooled, and grown for a further week in G418 before assay. Inducible luciferase activity has been maintained thereafter for several months in culture. For the IL-12 promoter assay,  $10^5$  cells were incubated in 1 ml medium overnight, followed by addition of ODN for 6 h, then lysis and assay as above.

#### Nitrite assay

The assay of nitrite using the Griess reagent was used as an indicator of NO production by both BMM and RAW264, as described previously (23). Briefly,  $4 \times 10^4$  RAW264 or  $10^5$  BMM (with  $10^4$  U/ml CSF-1) were plated in 96-well plates in 200  $\mu$ l supplemented RPMI and cultured overnight. Cells were then pretreated with 20 U/ml murine rIFN- $\gamma$  for 2 h (R&D Systems, Minneapolis, MI), followed by addition of the triggering stimulus. After 24 h, supernatants were removed and assayed for nitrite.

#### Cellular viability assay

The reduction of the dye MTT (Sigma) was used to measure relative number of viable cells. BMM were harvested and plated in CSF-1-free medium for 18 h ( $5 \times 10^4$  cells/well in 200  $\mu$ l in a 96-well plate). The desired additions were made, and the cells were incubated for an additional 48 h before assessment of MTT reduction, as described previously (32).

#### Down-modulation of surface CSF-1R

BMM were starved of CSF-1 overnight, then incubated with the desired treatments on bacteriological petri dishes, before being immunostained for surface expression of CSF-1R using the specific mAb AFS98, as described previously (32).

#### Phosphorylation of ERK-1/2

Cell extracts and Western blotting for the determination of the phosphorylation state of the extracellular signal-regulated kinases 1 and 2 (ERK-1/2) were conducted as described previously (32).

#### Intracellular TNF- $\alpha$ assays

For the plate assay, RAW264 cells were plated at  $10^5$  cells/well in 96-well plates and incubated overnight. To increase cellular retention of TNF- $\alpha$ , brefeldin A (10  $\mu$ g/ml) was added 15 min before incubation with ODN for 2 h. Cells were washed once in ice-cold PBS and fixed with 20% acetone, 0.02% BSA in PBS for 2 h at 4°C. Cells were washed three times with PBS, blocked for 1 h with 1% BSA in PBS, and then incubated for 1 h with 1/4000 dilution of rabbit anti-mouse TNF- $\alpha$  Ab (Genzyme, Cambridge, MA) in PBS/1% BSA. Cells were washed three times with 0.2% Tween 20 in PBS, for a total of 20 min, then incubated for 1 h with 1/1000 dilution of secondary Ab (anti-rabbit HRP conjugate; New England Biolabs, Beverly, MA) in PBS/1% BSA. Secondary Ab was removed and cells were incubated for 30 min in 5% goat serum in PBS/1% BSA. Cells were washed three times with 0.2% Tween 20 in PBS, for a total of 20 min, and then once in PBS. Color development with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) reagent (560  $\mu$ g/ml ABTS (Sigma), 0.008%  $H_2O_2$ , 50 mM citric acid, 0.1 M  $Na_2HPO_4$ , pH 4.2) was measured at 415 nm.

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For FACS analysis, RAW264 cells plated in 1 ml of medium were treated with 5  $\mu$ g/ml brefeldin A together with ODN for 4 h. Cells were harvested and stained for intracellular TNF- $\alpha$ , as described by Underhill et al. (30).

#### ODN and dextran uptake

RAW264 cells were plated overnight at 250,000/well in 1 ml of medium in 24-well plates, and reduced to 0.25 ml before addition of ODN. Cy3-labeled ODN were added for the indicated times before removal of supernatant and addition of ice-cold PBA (PBS/0.1% BSA/0.1% sodium azide). Cells were harvested, and incubated on ice for 10 min with 25 mg/ml dextran sulfate in PBA to remove surface-bound ODN. Cells were then washed twice in 5–10 ml PBA and analyzed by flow cytometry on a FAC-Scalibur (Becton Dickinson, San Jose, CA) for internalized ODN. Control samples in which cells were incubated with ODN on ice to allow cell surface binding but not internalization showed that the procedure did effectively strip off surface-bound ODN. Results were corrected for a slight difference in the degree of fluorochrome labeling of the two ODN, AO-1 and AOS-1. For fluorescein-labeled dextran uptake, cells were processed as for ODN uptake experiments, except incubation with dextran sulfate was omitted.

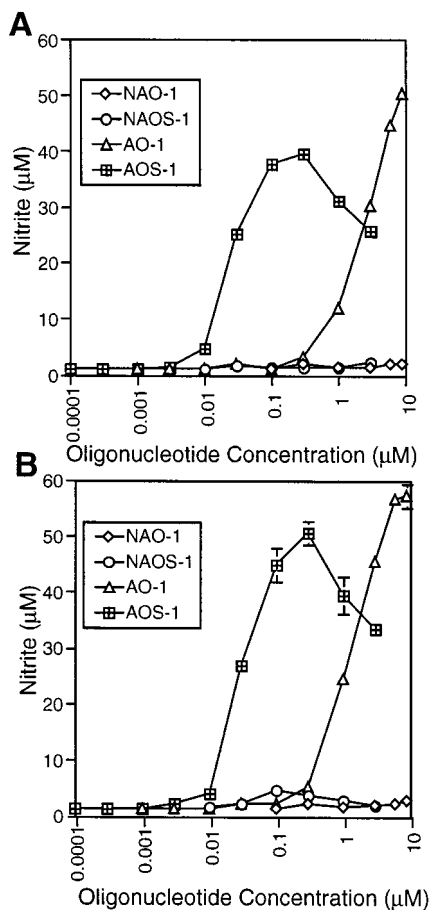
## Results

### *PS-ODN are more potent than PO-ODN in induction of NO, promotion of cell survival, and activation of the IL-12 promoter*

Macrophages primed with IFN- $\gamma$  respond to exposure to CpG DNA with expression of inducible NO synthetase mRNA and increased NO production (21, 23). PO-ODN and PS-ODN of the same sequence (AO-1 and AOS-1) were compared for their ability to induce NO production in IFN-primed BMM and RAW264 cells in Fig. 1, A and B, respectively. The dose-response curves for the primary cells and the cell line are almost indistinguishable. Both ODN were active, but AOS-1 was much more potent with responses detectable at  $\sim$ 100-fold lower concentration than for AO-1. The PS-ODN AOS-1 always gave a lower maximal activity than AO-1. High AOS-1 concentrations caused NO production to decline from the maximum. A second pair of PO- and PS-ODN, AAC-22 and SAAC-22, gave a similar difference in potency, and inhibition of response at high PS-ODN concentration (result not shown). CpG specificity was preserved for the response to AOS-1, although low levels of NO production were observed occasionally with the PS-ODN NAOS-1 (see Fig. 11), which differs only in having a reversal of the CpG motif to GpC.

Because the induction of NO production by CpG DNA depends upon IFN- $\gamma$  priming, we considered the possibility that IFN sensitized specifically to PS-ODN. We have shown elsewhere that CpG DNA can prevent apoptosis that occurs in BMM deprived of the macrophage growth and survival factor, CSF-1 (32). Fig. 2 compares the dose-response curves for the antiapoptotic activity of AO-1 and AOS-1. Assessment of viable cell number was based upon reduction of the dye MTT by the mitochondrial enzyme succinate dehydrogenase. Because this assay is sensitive to factors that alter mitochondrial respiration, we have previously validated it for this use by showing a good correlation between MTT reduction and direct cell count (32). AO-1 and AOS-1 both maintained cell viability in the absence of CSF-1, whereas the corresponding GpC transversions lacked activity. In comparative dose-response curves, AOS-1 was  $\sim$ 30-fold more active than AO-1 (Fig. 2). The dose-response curves for AOS-1 and AO-1 were similar in the cell viability and NO assays, suggesting that IFN- $\gamma$  does not sensitize preferentially to phosphorothioate CpG DNA. As both the NO



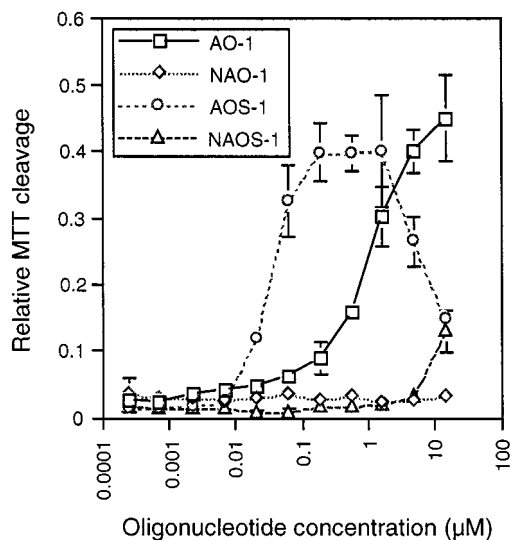


**FIGURE 1.** PS-ODN are more potent in induction of NO production than sequence identical PO-ODN. BMM (A) or RAW264 cells (B) were primed for 2 h with 20 U/ml IFN- $\gamma$ , then incubated for an additional 24 h with the indicated concentration of PS-ODN (AOS-1, NAOS-1) or PO-ODN (AO-1, NAO-1). Nitrite was measured in the supernatant. Data are the average and SD of triplicates. The experiment is representative of three.

assay and the antiapoptotic assay involve incubation for extended periods in culture, the first explanation considered for the higher activity of PS-ODN was their stability relative to PO-ODN. We therefore examined several shorter term assays, in which a smaller difference in activity of PS- and PO-ODN might be expected. Stimulation of an IL-12 promoter-reporter construct in RAW264 cells for 6 h gave a similar threshold for response to PO-ODN as the previous assays (Fig. 3). Again, the PS-ODN was active at much lower concentrations and had a lower maximal activity than the PO-ODN.

*PS-ODN are less active than PO-ODN in activation of the HIV-1 LTR and down-modulation of cell surface CSF-1R*

In previous work, we have measured NF- $\kappa$ B-dependent transcription in macrophages after 2-h exposure to CpG DNA, using a RAW264 cell line with an integrated HIV-1 LTR luciferase reporter construct (21). Using this assay (Fig. 4A), the PO-ODN AO-1 gave a similar dose-response curve to the longer term NO and antiapoptotic assays (Figs. 1 and 2). By contrast, the corresponding PS-ODN, AOS-1 was almost inactive. The same pattern was observed with the PO-ODN AAC-22 and its corresponding PS-ODN SAAC-22 (Fig. 4B). Both PS-ODN caused a minimal activation of the HIV-1 LTR at low concentration, but had little additional impact with increasing concentration.

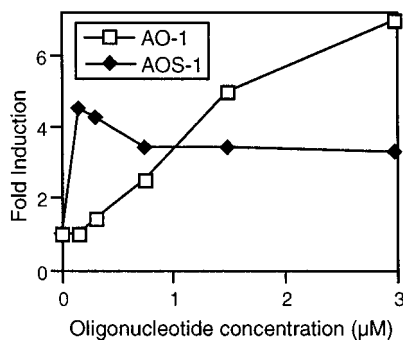


**FIGURE 2.** PS-ODN are more active than PO-ODN in maintenance of viability of BMM. BMM were starved of CSF-1 for 18 h, then treated with the indicated concentrations of PS-ODN (AOS-1, NAOS-1) or PO-ODN (AO-1, NAO-1) for an additional 48 h. Cleavage of the dye MTT to form an insoluble formazan product was measured as an indication of the number of viable cells. Results are the mean and SD of triplicates. The result is representative of three experiments.

BMM treated with either bacterial DNA, CpG PO-ODN, or LPS rapidly down-modulate cell surface expression of the CSF-1R, a response that occurs in an all-or-nothing manner at the single cell level (32). Dose-response analysis of CSF-1R down-modulation was conducted with AOS-1 and AO-1 (Fig. 5, A and B). Fig. 5A shows the representative flow-cytometric profiles. Increasing AO-1 concentration increased the proportion of cells with completely down-modulated CSF-1R (falling within the M1 gate). Similar to the observations made with activation of the HIV-1 LTR, PS-ODN were much less active than their PO-ODN counterparts. After a 1-h incubation with ODN at a concentration of 3  $\mu$ M, AOS-1 increased the percentage of cells with receptor down-modulation from 12% to 25%, whereas with AO-1 95% of cells fully down-modulated their CSF-1R. The same trend was observed in a comparison of SAAC-22 and AAC-22 (Fig. 5C).

*PS-ODN give delayed and reduced phosphorylation of ERK-1/2*

Maintenance of cell viability in CSF-1-starved BMMs is associated with the ability of both LPS and CpG DNA to activate the MAP kinases, ERK-1/2 (32). LPS causes rapid activation of the MAP/ERK kinase-1, which in turn phosphorylates ERK-1/2. A stimulatory ODN such as AO-1 acts more slowly than LPS, perhaps reflecting the time required for ODN internalization (unpublished observation). Fig. 6A compares the time course of activation of ERK-1/2 in BMM by AO-1 and AOS-1. AO-1 caused maximal phosphorylation of ERK-1/2 by 20 min, followed by a decline to an elevated steady state after 40 min. By contrast, AOS-1 showed no induction of ERK-1/2 phosphorylation at 20 min, and reached a peak at 1 h that was less than the maximal phosphorylation observed with AO-1. Both ODN treatments in Fig. 6A were 3  $\mu$ M. Because AOS-1 seems to have some self-inhibitory action at this concentration (Figs. 1 and 2), we checked whether lower concentrations could induce earlier phosphorylation of ERK-1/2. Fig. 6B shows that concentrations of AOS-1 between 0.1 and 1  $\mu$ M were also unable to stimulate ERK-1/2 phosphorylation at 20 min. We found a similar delay in ERK-1/2 phosphorylation in response to AOS-1 in RAW264 cells (result not shown).



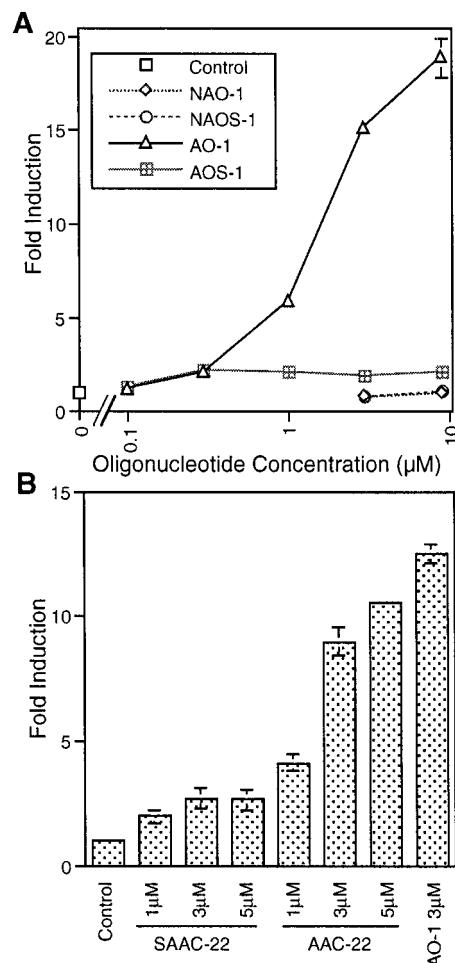
**FIGURE 3.** PS-ODN are more active than PO-ODN in activation of the IL-12p40 promoter. RAW264 cells stably transfected with a mouse IL-12 promoter-luciferase reporter were incubated for 6 h with the indicated concentrations of ODN before harvesting for determination of luciferase activity. Results are the average of duplicates, and the experiment is representative of three.

#### PS-ODN are taken up more rapidly than PO-ODN

Delayed ERK-1/2 phosphorylation, poor activation of the HIV-1 LTR at 2 h, and little down-modulation of the CSF-1R at 1 h by PS-ODN suggested a slower cellular activation by PS-ODN than PO-ODN. Cellular activation by CpG DNA apparently requires DNA uptake (20). Ineffective activation by PS-ODN in some short-term assays might potentially be explained by slower uptake into the cell. Previous studies have shown PO-ODN to be more rapidly taken up than PS-ODN in a colorectal adenocarcinoma cell line (33), but faster uptake of PS-ODN in renal epithelial cells (34) and B cells (35). To check whether poor responses were due to slower uptake of PS-ODN, we measured cellular accumulation of Cy3-labeled PS- and PO-ODN by flow cytometry. Cy3 is not pH sensitive between pH 5 and 7.5 and is therefore unaffected by endosomal acidification. Fig. 7 shows that PS-ODN actually accumulated much more rapidly than PO-ODN in RAW264 cells. The slower cellular accumulation of PO-ODN seen in this study was not due to its extracellular degradation, because the differential uptake was maintained in cells freshly plated in serum-free medium (result not shown). Given that the difference in rates of uptake was apparent after only 5 min, it is also not likely to be due to intracellular degradation of PO-ODN and exocytosis of the Cy3 label. Thus, slow uptake of PS-ODN is not responsible for its poor activity in some assays. The possibility remains that PS-ODN take longer to reach the site of detection of CpG DNA within the cell.

#### PS-ODN efficiently induce TNF- $\alpha$

Although PS-ODN did not efficiently activate macrophages in two other assays performed after 1 and 2 h of stimulation (Figs. 4 and 5), it did induce TNF- $\alpha$  protein in a 2-h incubation (Fig. 8A). The response to low concentrations of PS-ODN was less sensitive than in the longer term NO and apoptotic assays, with first responses seen at 0.1  $\mu$ M rather than 0.03  $\mu$ M. Nevertheless, any delay in signaling in response to PS-ODN is insufficient to prevent induction of TNF- $\alpha$  at 2 h. Analysis at the single cell level using flow cytometry showed that AOS-1 induced lower levels of TNF- $\alpha$  in individual cells than AO-1 (Fig. 8, B and C). In addition, although all cells responded to 3  $\mu$ M AO-1, a population of cells failed to be stimulated by the PS-ODN AOS-1. The lower peak response, and decline in activity at high PS-ODN concentration seen in this study (Fig. 8A) and in other assays (Figs. 1, 2, and 3) suggested that PS-ODN have an inhibitory action at high concentration. Given this, we investigated whether this inhibition was an inherent

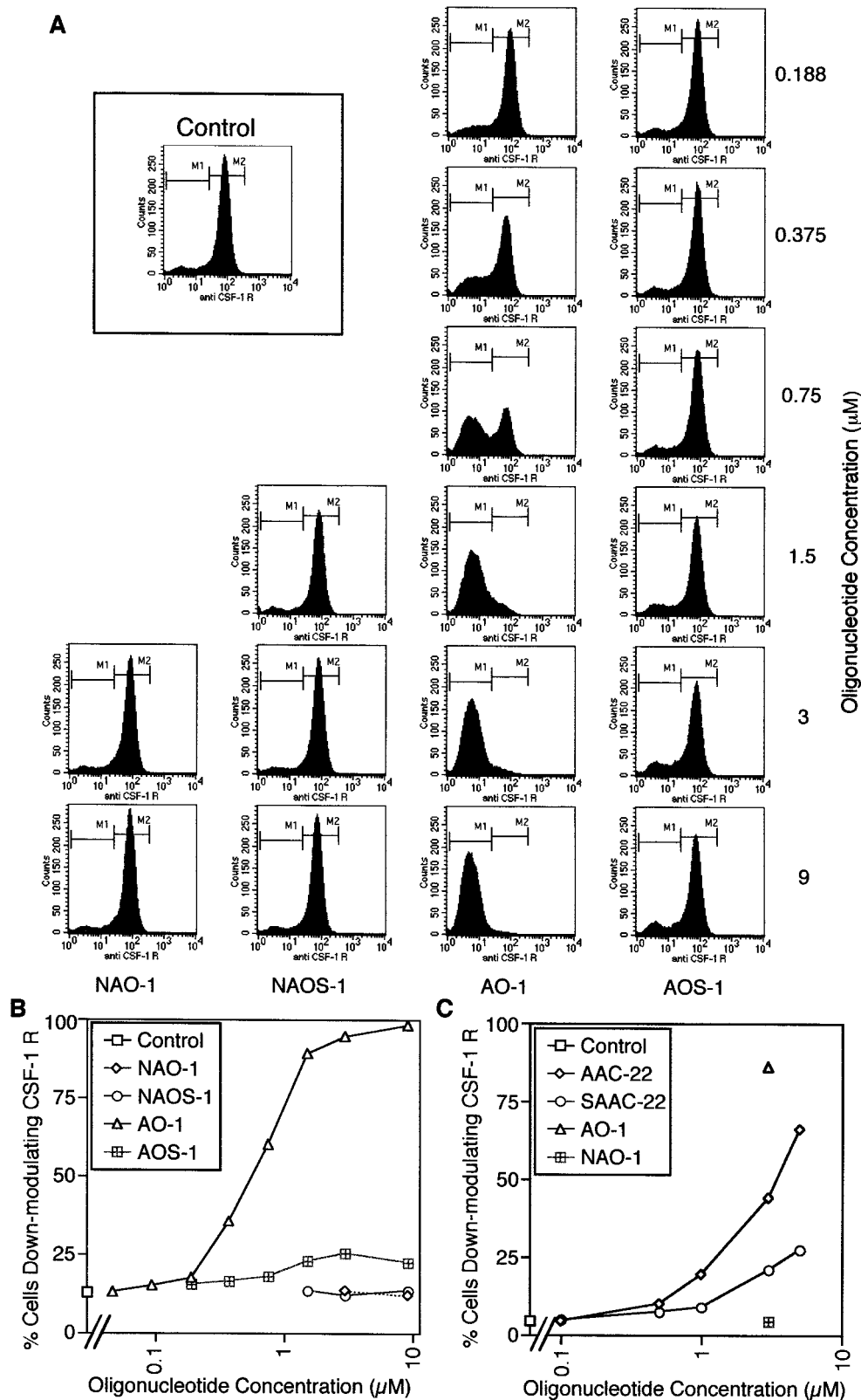


**FIGURE 4.** CpG PS-ODN do not activate the HIV-1 LTR. RAW264a4 cells, with an integrated HIV-1 LTR-luciferase reporter gene, were incubated for 2 h with the ODN at the indicated concentrations before being harvested for determination of luciferase activity. The activity observed in unstimulated cells is arbitrarily given a value of 1. Results are the average and SD of triplicates. The experiments are representative of at least three.

property of PS-ODN and whether non-CpG PS-ODN can block the CpG response.

#### Blockade of CpG DNA responses by non-CpG PS-ODN

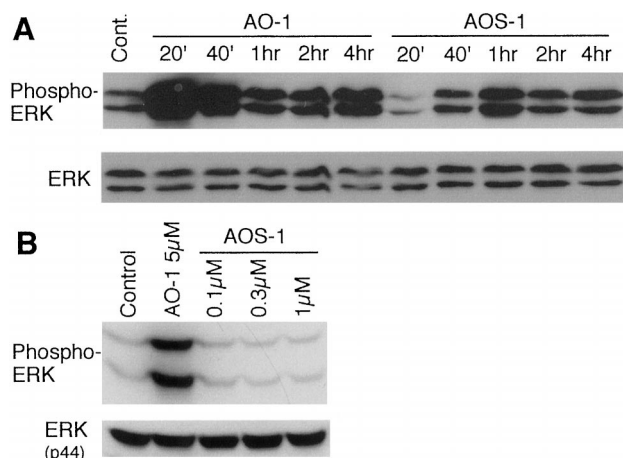
Häcker et al. (36) have presented evidence that non-CpG PS-ODN can block the response to CpG PS-ODN. Whether the same applies for PO-ODN has not previously been addressed. Preincubation or cotreatment with an equimolar concentration (2  $\mu$ M) of the inactive PO-ODN NAO-1 had no significant effect on activation of HIV-1 LTR-dependent luciferase activity in RAW264 cells induced by AO-1. Both pre- and cotreatment with an equimolar concentration of the equivalent PS-ODN NAOS-1 substantially diminished activation by AO-1 (Fig. 9A). Even a 6-fold molar excess of the non-CpG PO-ODN NAO-1 had no effect on the response to AO-1 at a concentration that was near saturating in biological CpG activity (Fig. 9B). The effect of non-CpG PS-ODN was specific in that it reduced CpG DNA-mediated activation of the HIV-1 LTR, but not activation mediated by LPS (Fig. 9A). Because even the CpG-containing PS-ODN were inactive in the HIV-1 LTR assay, we examined whether they might also be inhibitory. Indeed, SAAC-22 was also able to prevent activation by AO-1 and AAC-22 (not shown). These findings indicate that PS-ODN have a dominant repressive effect on the ability of CpG DNA, but not LPS to activate in this assay.



**FIGURE 5.** CpG PS-ODN fail to down-modulate the CSF-1R from the cell surface of BMM. BMM were starved of CSF-1 overnight, then incubated with the indicated concentrations of ODN for 1 h and immunostained for surface expression of CSF-1R. **A**, Representative FACS profiles from one experiment. **B**, Analysis of the proportion of cells showing down-modulated CSF-1R (the M1 fraction shown in **A**) in response to the indicated ODN concentrations. **C**, Analysis of the proportion of cells showing down-modulated CSF-1R in a separate experiment with PO-ODN AAC-22 and PS-ODN SAAC-22.

The time course of PS-ODN-specific blockade of HIV-1 LTR activation by AO-1 was investigated in Fig. 10. There was no requirement for pretreatment; in fact, the extent of inhibition was

greatest when PS-ODN were coadministered, whereas there was little effect where it was added only 15 min after treatment with AO-1. The lack of inhibition following delayed addition is



**FIGURE 6.** PS-ODN induce phosphorylation of ERK-1/2 more slowly than PO-ODN. BMM were starved of CSF-1 overnight, then incubated for the indicated times with AO-1 or AOS-1 before lysis and Western blotting to detect ERK-1/2 phosphorylation. A representative experiment is shown. *A*, Cells were treated with 3  $\mu$ M AO-1 or AOS-1 for various times. *B*, Cells were treated with 0.1, 0.3, or 1  $\mu$ M AOS-1, or 5  $\mu$ M AO-1 for 20 min.

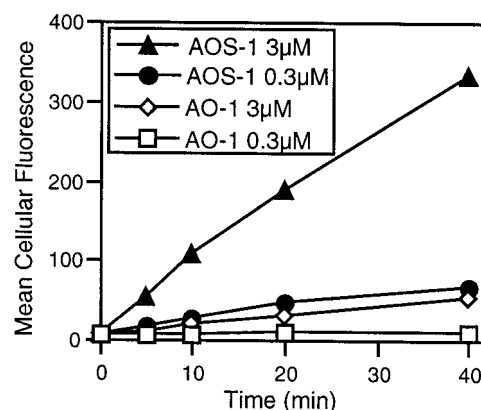
consistent with the pattern of activation of ERK-1/2 (see Fig. 6), which is already maximal after 20 min. High dose inhibition observed in the dose-response curve for PS-ODN activation of NO production could also reflect a compromise between stimulation by the CpG motif, and inhibition by the backbone. This possibility was investigated by preincubating cells with non-CpG ODN. As seen in the HIV-1 LTR assay, nonstimulatory PS-ODN blocked the response to AO-1, whereas minimal inhibition of the LPS response was observed (Fig. 11). Responses to the PS-ODN AOS-1 and SAAC-22 were also inhibited by non-CpG PS-ODN (result not shown). By contrast, there was no inhibition by the corresponding non-CpG PO-ODN (NAO-1) (Fig. 11) even at concentrations of 10  $\mu$ M (result not shown).

Häcker et al. (36) have shown inhibition of TNF- $\alpha$ , IL-6, and IL-12 production in a macrophage cell line by non-CpG PS-ODN. They also showed by fluorescence microscopy that at a concentration of 3  $\mu$ M, non-CpG PS-ODN blocked the uptake of an active PS-ODN at 1  $\mu$ M. We examined the effect of pretreatment with PS- or PO-ODN on the uptake of Cy3-labeled ODN (Fig. 12). The PO-ODN NAO-1 at 9  $\mu$ M had minimal effect on ODN uptake. Increasing concentrations of NAOS-1 reduced the uptake of Cy3-AOS-1. However, although 9  $\mu$ M NAOS-1 reduced the uptake of the PO-ODN Cy3-AO-1 by 50%, at lower concentrations NAOS-1 had no effect or was even stimulatory. Thus, inhibition of uptake does not account for the inhibition of activity of AO-1 seen with NAOS-1 between 1 and 3  $\mu$ M (Figs. 9 and 11). NAOS-1 did not inhibit uptake of fluorescein-labeled dextran (Fig. 12), indicating that PS-ODN had no effect on fluid-phase uptake in macrophages.

## Discussion

Interest in studying the responses to CpG-containing DNA arises from several distinct biological applications. In gene therapy, DNA vaccination, and the possible roles of CpG DNA sequences in host-pathogen interaction, the active molecules are native phosphodiester DNAs. Applications of CpG DNA in immunotherapy and antisense therapy are based on phosphorothioate-modified backbones. In this study, we have shown that PS-ODN have quite distinct activities from PO-ODN in the activation of macrophages.

The phosphorothioate modification of ODN is necessary for effective immunomodulatory effects to be seen in vivo. The general

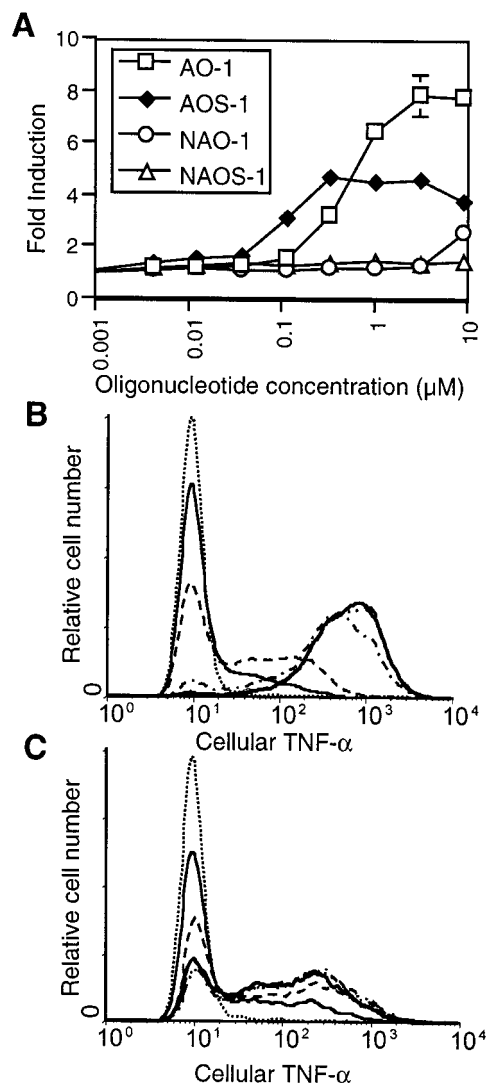


**FIGURE 7.** PS-ODN are taken up more rapidly than PO-ODN. RAW264 cells were incubated with 0.3 or 3  $\mu$ M Cy3-labeled AOS-1 or AO-1 for various times. ODN uptake as measured by cellular fluorescence was determined by flow-cytometric analysis.

assumption is that this is due to the increase in stability of the ODN, as PO-ODN are rapidly degraded in vivo (11). PS-ODN also have significantly greater stability in vitro. Matsukura et al. (37) found the  $t_{1/2}$  of PO-ODN in medium with 15% FCS to be 17 h, while PS-ODN showed no degradation after 7 days. Another report found PO-ODN within spleen cells was partially degraded after 4-h incubation, although PS-ODN was intact (35). Even after 72-h incubation, PS-ODN was 90% intact in cells and culture medium (38). If CpG PS-ODN behaved just like PO-ODN but with greater stability, then we would expect that long-term assays would show the greatest disparity in activity between the two types of ODN. There would be a fairly constant response to PS-ODN in different assays and a declining relative potency of PO-ODN as the time of incubation increased. This is not what we have observed. Regardless of the length of incubation, the dose response for PO-ODN in a range of assays was fairly similar, with activity evident at concentrations above 0.3–1  $\mu$ M (Figs. 1–5 and 8). The effects of the phosphorothioate modification varied widely between assays. Responses to PS-ODN were evident at 10–100-fold lower concentrations than PO-ODN for induction of NO, activation of the IL-12 promoter, and prevention of cell death (Figs. 1–3). The much greater potency of PS-ODN in these assays, and in in vivo immunomodulation is likely to be due not only to greater stability, but also to much more efficient uptake of PS-ODN (Fig. 7). However, CpG PS-ODN were much less potent than PO-ODN in down-modulation of surface expression of the CSF-1R, almost inactive in activation of the HIV-1 LTR, and gave delayed and reduced ERK-1/2 phosphorylation (Figs. 4–6).

The poor responses to PS-ODN seen in some of the shorter assays were not due to slow uptake of PS-ODN. The possibility remained that PS-ODN is slower in getting to the site of recognition of foreign DNA, which remains uncharacterized. Endosomal release could be a rate-limiting step for responses to CpG DNA. The effective response to PS-ODN in induction of TNF- $\alpha$  protein by 2 h (Fig. 8A) suggests that this is not the major issue. In addition, extension of the HIV-1 LTR assay to 6 h failed to give efficient activation by PS-ODN, and treatment of CSF-1-deprived BMM for 12 h with PS-ODN resulted in only 50% of cells down-modulating surface expression of the CSF-1R (results not shown). Thus, there seems to be a qualitatively different response to the PS-ODN, rather than merely a delayed response. A remaining possibility for poor activation in some assays is that PS-ODN give a selective inhibition or lack of activation of certain signaling pathways. Coordinated activation of signaling pathways may be important in some responses, and a delay in activation of pathways

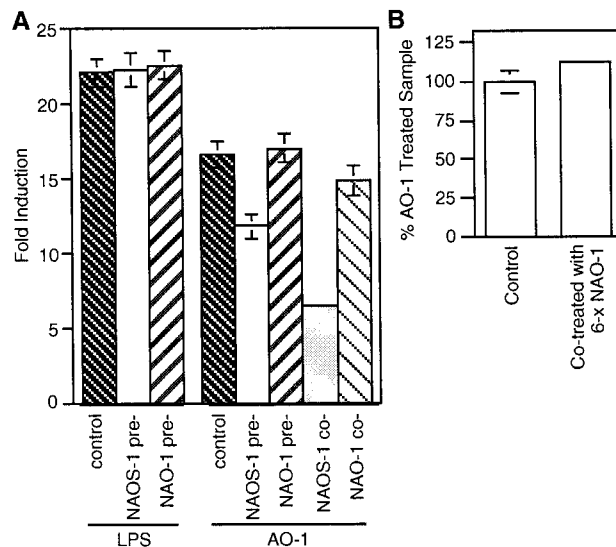




**FIGURE 8.** PS- and PO-ODN induce TNF- $\alpha$ . *A*, Intracellular TNF- $\alpha$  was measured in RAW264 cells by a plate assay. Cells were incubated with various concentrations of ODN for 2 h. Results show the average and SD of triplicate estimations, and are representative of three experiments. *B*, Intracellular TNF- $\alpha$  was measured by flow-cytometric analysis. Cells were incubated with AO-1 at 0, 0.075, 0.15, 0.75, 1.5, and 3  $\mu$ M for 4 h. Unstimulated cells are shown as a dotted line. Increasing concentrations of ODN are shown, in order, by thin solid line, dashed line, dash-dot, dash-dot-dot, with the highest concentration a thick solid line. *C*, Intracellular TNF- $\alpha$  measured as per *B*, using the PS-ODN, AOS-1.

such as the ERK MAP kinases (Fig. 6A) may prevent responses. In addition, a lowered level of activation of signaling pathways, as observed for ERK-1/2 with PS-ODN, may stimulate some responses and not others, if the responses have different thresholds for activation. A comparison of PS- and PO-ODN activation of other pathways such as NF- $\kappa$ B, JNK, and p38 will be informative. Others have reported that CpG PS-ODN can induce NF- $\kappa$ B nuclear translocation in RAW264 cells (25). Thus, the failure of PS-ODN to stimulate the HIV-1 LTR (Fig. 4) suggests that PS-ODN may poorly activate some of the other transcription factors required, such as Ets, Sp-1, or PU.1 (39, 40).

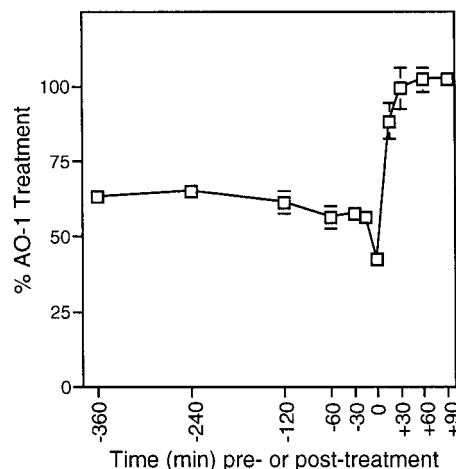
Another way in which the two types of ODN vary is the sequence-independent inhibitory activity of PS-ODN seen at high concentrations. Non-CpG PS-ODN at concentrations of 1  $\mu$ M and above inhibited responses to both CpG PO-ODN (Figs. 9 and 11)



**FIGURE 9.** PS-ODN inhibit activation of the HIV-1 LTR by CpG DNA. RAW264a4 cells, with integrated HIV-1 LTR-luciferase reporter gene, were treated with AO-1 or LPS for 2 h before harvesting and determination of luciferase. Unstimulated luciferase activity is given an arbitrary value of 1. *A*, Cells were incubated with 2  $\mu$ M AO-1 or 10 ng/ml LPS for 2 h. They were either pretreated for 2 h, or cotreated with 2  $\mu$ M non-stimulatory PO-ODN (NAO-1) or PS-ODN (NAOS-1). *B*, Cells were incubated with AO-1 at 5  $\mu$ M, which gives a near maximal response, with or without 6-fold excess (30  $\mu$ M) NAO-1. Data shown are the mean and SD of triplicates.

and PS-ODN (result not shown). This concentration coincides with the threshold above which CpG PS-ODN exhibit a self-inhibitory activity (Figs. 1 and 2). Inhibition of responses to PS-ODN at high concentrations has been observed previously (17). The inhibition seen in this study was not due to an intrinsic toxicity of high concentrations of phosphorothioate, as LPS responses were not greatly inhibited (Figs. 9 and 11). A previous study has suggested that non-CpG PS-ODN may inhibit CpG responses by blocking ODN uptake (36). We found some inhibition of uptake of labeled ODN after treatment with non-CpG PS-ODN, which was not seen with high concentrations of non-CpG PO-ODN (Fig. 12). The degree of inhibition of uptake was not sufficient to account for the inhibition of CpG activity. Thus, we propose that PS-ODN have two inhibitory activities at high concentration, one that reduces the uptake of ODN, and another uncharacterized activity accounting for the self-inhibition seen at higher concentrations with CpG PS-ODN.

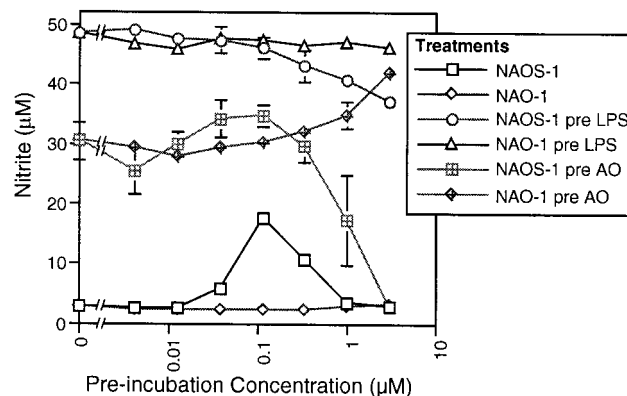
Non-CpG PS-ODN inhibited the uptake of labeled CpG PS-ODN in a dose-dependent manner, but was less effective at inhibiting the uptake of labeled PO-ODN (Fig. 12). This, together with the much poorer uptake of PO-ODN, suggests a difference in the mechanism of uptake between the two types of ODN. In K562 human leukemia cells, PS-ODN uptake was primarily receptor mediated at concentrations below 1  $\mu$ M, with fluid-phase uptake becoming important at higher concentrations (41). A number of studies have looked for DNA receptors on the cell surface, but the mechanism of ODN uptake remains to be established (20, 42). The integrin MAC-1 (CD18/CD11b) has been implicated as a specific receptor for ODN in neutrophils (43). We found no evidence to support this function in murine macrophages, in which ODN treatment did not alter the level of MAC-1 Ag on the cell surface (32). In addition, cells from mice deficient in MAC-1 have normal ODN uptake (20).



**FIGURE 10.** Inhibition of CpG DNA response by PS-ODN does not require preincubation. RAW264a4 cells, with integrated HIV-1 LTR-luciferase reporter gene, were treated with 2  $\mu$ M AO-1 for 2 h before harvesting for measurement of luciferase activity. The non-CpG PS-ODN NAOS-1 was added at equimolar concentration at the times indicated relative to the addition of AO-1. Results are the average and SD of triplicates.

In preliminary binding studies with murine macrophages, we found that PO-ODN had much lower cell surface binding than PS-ODN (result not show). This has also been found using spleen cells (35). Because PO-ODN binding to spleen cells is efficiently competed for by PS-ODN (35), it is unlikely that they are binding completely different classes of receptor. The differences in rate of uptake, surface binding, and inhibition of uptake are consistent with PS-ODN binding and uptake by a high affinity receptor, and much poorer receptor-mediated uptake of PO-ODN. With low receptor-mediated uptake, the contribution of fluid-phase uptake of PO-ODN would become relatively more important, explaining the poor inhibition of PO-ODN uptake by PS-ODN (Fig. 12). The absence of an effect of PS-ODN on uptake of labeled dextran indicates that fluid-phase uptake is not inhibited by PS-ODN (Fig. 12). Because receptor-mediated uptake is inherently saturable, the inhibition of labeled CpG PS-ODN uptake by non-CpG PS-ODN is likely to be due to competition for receptor binding. Lack of inhibition by high concentrations of PO-ODN is explained by its lower affinity for the receptor.

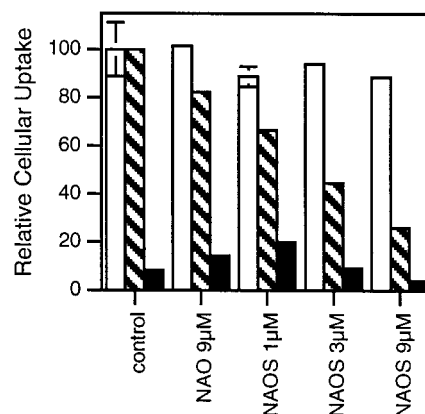
In addition to the inhibitory activities we have observed for PS-ODN, sequence-independent activation has been documented. In vivo administration of PS-ODN caused splenomegaly and B cell proliferation, which was enhanced by the presence of CpG motifs (18). Sequence-independent activation of human B cells (19) and enhancement of LPS-induced TNF- $\alpha$  production (44) are thought to be mediated by PS-ODN interaction with cell surface molecules. Activation of Sp1 transcription factor has also been observed (45). Some of these effects may be explained by the enhanced affinity of PS-ODN for binding to a wide variety of proteins (46, 47). For example, PS-ODN binding inhibits isoforms of protein kinase C (48), and can interfere with binding of growth factors to their receptors (49). Such interactions with signaling molecules could be responsible for the lack of activation by PS-ODN of some macrophage responses to CpG DNA, as well as the inhibitory effects we have observed at high concentration. Another effect of the phosphorothioate modification may be to constrain the three-dimensional structure of the ODN and affect its interaction with the putative CpG receptor. Whether this could have different effects on different responses, as we have observed in this study, remains to be established. The poor activity of PS-ODN in eliciting some



**FIGURE 11.** PS-ODN prevent activation of NO production induced by CpG DNA. RAW264 cells were primed with 20 U/ml IFN- $\gamma$  for 2 h, then treated for 24 h with 1  $\mu$ M AO-1 or 5 ng/ml LPS, as indicated. Non-CpG PO-ODN NAO-1, or PS-ODN NAOS-1 were added at the concentrations shown, either alone, or 30 min before LPS or AO-1. Results are the mean and SD of triplicates, and are representative of at least three experiments.

responses could be somewhat sequence dependent. Krieg et al. (17, 20) have suggested that the sequence requirements for activity are more stringent with PS-ODN than with PO-ODN, and another group has shown a sequence-dependent lack of activity of PS-ODN in induction of TNF- $\alpha$  (50). Thus, the change in ODN structure introduced by the phosphorothioate modification seems to have a greater effect on the immunostimulatory ability of some sequences than others.

In summary, we have demonstrated that the phosphorothioate backbone modifies the response to activating CpG sequences in a number of ways. The phosphorothioate modification that makes ODN resistant to nuclease attack also greatly enhanced their uptake into murine macrophages. The increased stability and uptake are likely to be responsible for the higher potency of PS-ODN in some assays. However, the PS-ODN failed to efficiently stimulate some responses. Delayed or poor activation of signaling components, such as the ERK MAP kinases, may contribute to this effect. In addition, PS-ODN have a CpG-selective inhibitory action at



**FIGURE 12.** High concentrations of PS-ODN reduce labeled ODN uptake. RAW264 cells were incubated with 0.25 mg/ml fluorescein-labeled dextran ( $\square$ ), 1  $\mu$ M Cy3-labeled AOS-1 ( $\square$ ), or 1  $\mu$ M Cy3-labeled AO-1 ( $\blacksquare$ ) for 40 min. They were pretreated for 30 min with the indicated concentrations of NAO-1 or NAOS-1. ODN uptake was measured by FACS analysis. Results are representative of three experiments and show the mean of duplicates. Error bars show the values of the duplicates. Results for ODN uptake are shown relative to the control Cy3-AOS sample. Dextran results were normalized to the control sample.

high concentrations that is only partially accounted for by inhibition of ODN uptake. Thus, PS-ODN do not perfectly mimic a biological response to natural CpG DNA. Whether this is an advantage or disadvantage in therapeutic applications remains to be established. Other backbone modifications may give qualitatively different responses of use in immunotherapy.

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